HIV IMMUNOGENIC COMPLEXES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Serial No. 09/905,962 filed on July 17, 2001, which is a continuation of U.S. Serial No. 09/479,675 filed on January 7, 2000, now U.S. Patent No. 6,328,973, which is a divisional of U.S. Serial No. 09/075,544 filed on May 11, 1998, now U.S. Patent No. 6,030,772, which is a divisional of U.S. Serial No. 08/464,680 filed on December 20, 1995, now U.S. Patent No. 5,843,454, which is a 371 of PCT/US94/05020 filed on May 6, 1994, which is a continuation-in-part of U.S. Serial No. 08/060,926 filed on May 7, 1993, now abandoned, all of which are hereby incorporated by reference herein.

DESCRIPTION OF THE INVENTION

We have discovered that a gp120-CD4 covalently bonded complex presents a specific subset of cryptic epitopes on gp120 and/or CD4 not present on the uncomplexed molecules. This complex elicits neutralizing antibodies with novel specificities and is thus useful in vaccines and immunotherapy against HIV infection. We have also discovered that complexes including gp120 covalently bonded to a fragment of CD4 elicit neutralizing antibodies and are therefore useful in vaccines and immunotherapy against HIV infection. In addition, these complexes or antibodies thereto can be used in immunological tests for HIV infection.

BACKGROUND OF THE INVENTION

Neutralizing antibodies are considered to be essential for protection against many viral infections including those caused by retroviruses. Since the initial reports of neutralizing antibodies in HIV-infected individuals, it has become increasingly clear that high levels of these antibodies in serum correlate with better clinical outcome (3 – 5). These studies suggested that the identification of

epitopes that elicit high titer neutralizing antibodies would be essential for vaccine development against HIV infection.

The primary receptor for the human immunodeficiency virus type 1 (HIV-1) is the CD4 molecule, found predominantly on the surface of T-lymphocytes. The binding of HIV-1 to CD4 occurs via the major viral envelope glycoprotein gp120 and initiates the viral infection process.

Current strategies for developing vaccines against infection by the human immunodeficiency virus have focused on eliciting antibodies against the viral envelope glycoprotein gp120 or its cell surface receptor CD4. Purified gp120 typically elicits type specific neutralizing antibodies that are reactive against epitopes that vary among virus isolates. This characteristic has hindered the use of gp120 as a vaccine.

CD4 has also been considered as a major candidate for development of a vaccine against HIV-1. Recent studies have demonstrated that sCD4 elicits HIV-1 neutralizing antibodies in animals and prevents the spread of infection in SIV-infected rhesus monkeys (1). However, autoantibodies to CD4 may themselves create immune abnormalities in the immunized host if they interfere with normal T-cell functions. Neutralizing antibodies against gp120 are elicited in vivo in HIV-1-infected individuals and can be elicited in vitro using purified envelope glycoprotein. However, gp120 contains five hypervariable regions one of which, the V3 domain, is the principal neutralizing epitope. Hypervariability of this epitope among strains is a major obstacle for the generation of neutralizing antibodies effective against diverse strains of HIV-1. For these reasons it has been believed that vaccine strategies using either purified CD4 or gp120 present several disadvantages.

We have overcome the shortcomings of type specific anti gp120 antibodies and antibodies against CD4 by raising anti-HIV-1 neutralizing antibodies using as the immunogen a complex of gp120 chemically coupled to either soluble CD4, the mannose-specific lectin succinyl concanavalin A (SC) or an equivalent thereof. We have found that these compounds induce similar conformational changes in gp120. The complexed gp120 appears to undergo a

conformational change that presents an array of epitopes that were hidden on the uncomplexed glycoprotein (2). A portion of such epitopes elicit group-specific neutralizing antibodies, which are not strain limited like the type specific antibodies discussed above. We have discovered that the covalently bonded CD4-gp120 complexes are useful for raising neutralizing antibodies against various isolates of HIV-1 and against HIV-2.

The major research effort in the development of subunit vaccines against HIV has been directed toward the envelope glycoprotein of the virus. Inoculation of gp160 or gp120 into animals elicits neutralizing antibodies against HIV (3, 4). The principal neutralizing epitope on gp120 has been located between amino acids 306 and 326 in the third variable domain (V3 loop) of the protein (4). This epitope has been extensively studied by using both polyclonal and monoclonal antibodies (3, 4). In most cases antibodies directed to this region neutralize HIV-1 in an isolate specific manner although there is evidence that a weakly neutralizing species of anti-V3 loop antibodies can cross-react with diverse isolates (8). The reason for type specificity of anti-V3 loop antibodies is the extensive sequence variability among various isolates. Prolonged culturing of HIV-infected cells with type specific anti-V3 loop antibodies induces escape mutants resistant to neutralization (9).

In addition to the V3 loop, other neutralizing epitopes encompassing genetically conserved regions of the envelope have been identified (10, 11). However, immunization against these epitopes elicits polyclonal antisera with low neutralizing titers (12). For example, the CD4 binding region of gp120, encompassing a conserved region, elicits neutralizing antibodies against diverse isolates (13). However, this region is not normally an immunodominant epitope on the glycoprotein.

The interaction of gp120 with CD4 has been studied in considerable detail and regions of the molecules involved in complex formation have been determined (14-16). There are now several lines of evidence that interactions with CD4 induce conformational changes in gp120. First, recombinant soluble CD4 (sCD4) binding to gp120 increases the susceptibility of the V3 loop to

monoclonal antibody binding and to digestion by exogenous proteinase (2). Second, sCD4 binding results in the dissociation of gp120 from the virus (17, 18). These conformational changes in gp120 are thought to facilitate the processes of virus attachment and fusion with the host cell membrane (2). Immunization with soluble CD4 and recombinant gp120, complexed by their natural affinity but not covalently bonded, resulted in the production of anti CD4 antibodies (31). Several murine monoclonal antibodies have been developed by immunization with mixtures of recombinant gp120 and sCD4 (31, 32). Antibodies raised in these studies were not strictly complex-specific and reacted with free gp120 or CD4; the neutralizing antibodies reacted with free sCD4, although they displayed various degrees of enhanced reactivity in the presence of gp120. The complexes used in these studies were unstable and comprised noncovalently bound gp120 and CD4.

A variety of N-linked carbohydrate structures of high mannose, complex and hybrid types present on the gp120 molecule may also play a role in the interaction of gp120 with host cell membranes (19-21). Indeed, a carbohydrate-mediated reactivity of gp120 has already been demonstrated with a serum lectin, known as mannose-binding protein, which has also been shown to inhibit HIV-1 infection of CD4+ cells (22). An additional carbohydrate-mediated interaction of gp120 has been shown with the endocytosis receptor of human macrophage membranes (21). It has been postulated that high affinity binding of accessible mannose residues on gp120 to the macrophage membrane may lead to virus uptake by the macrophage (21).

Recombinant soluble CD4 has been shown to inhibit HIV infection in vitro, mainly by competing with cell surface CD4. This observation has led to the possibility of using sCD4 for the therapy of HIV-infected individuals (23, 24). In addition, sCD4 has been used as an immunogen to block viral infection in animals. Treatment of SIV_{MAC}-infected rhesus monkeys with sCD4 elicited not only an antibody response to human CD4 but also to monkey CD4. Coincident with the generation of such immunological responses, SIV could not be isolated from the PBMC and bone marrow macrophages of these animals (1). A recent

study with chimpanzees also demonstrated that human CD4 elicited anti-self CD4 antibody that inhibited HIV infection in vitro (25). Although immunization with sCD4 may be beneficial in blocking HIV infection, circulating antibody that recognizes self antigen may induce immune abnormality and dysfunction by binding to uninfected CD4+ cells. Nevertheless in theory anti-CD4 antibodies could be effective in blocking HIV infection provided they can disrupt virus attachment and entry without interfering with normal CD4 function. Ideally these antibodies should recognize CD4 epitopes that are present only after interaction with gp120.

The present invention overcomes the shortcomings in the art by providing for complexes of gp120 chemically coupled to either CD4, SC or an equivalent thereof. However, in some instances such as immunization of small animals as well as non-human primates, a high level of CD4 antibodies in the immunized host is not desirable since such antibodies may influence the immunological functions of T cells expressing CD4. Complexes with optimized immunological properties which elicit high levels of anti-gp120 and anti-complex antibodies with reduced levels of anti-CD4 antibodies following immunization are needed in such instances and the present invention also provides such complexes. In particular, the present invention provides complexes including gp120 chemically coupled with a fragment of CD4 or an equivalent thereof.

SUMMARY OF THE INVENTION

We have discovered that gp120-CD4 complex formation induces a specific subset of cryptic epitopes on gp120 and/or CD4 not present on the uncomplexed molecules. These epitopes elicit neutralizing antibodies with novel specificities and are thus useful in vaccines and/or immunotherapy of patients infected with HIV. In addition, the antibodies or the complexes can be used in immunological tests for HIV infection. We have demonstrated that the lectin, SC, mediates changes in the structure of gp120 in a manner similar to that mediated by CD4. The binding of SC to gp120 is another mechanism for inducing novel epitopes on the viral glycoprotein. The binding of other CD4 equivalent

molecules to gp120 is also another mechanism for inducing epitopes on the viral glycoprotein.

We used chemically coupled gp120-CD4 complexes as immunogens for raising neutralizing antibodies. We found that gp120-CD4 complexes possess novel epitopes that elicit neutralizing antibodies. Coupling with SC caused perturbation in the gp120 conformation which in turn unmasked cryptic neutralizing epitopes on gp120.

We have also discovered that covalently cross-linked complexes including gp120 and a fragment of CD4 or an equivalent thereof, elicit a broad anti-HIV response and are therefore useful in vaccines and immunotherapy against HIV infection.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B show the dissociation of gp120 from HIV-1 in the presence of sCD4 and SC. In Figure 1A, labeled cells were treated with 0 (lanes 1, 2) or 1.5 μg/ml sCD4 (lanes 3, 4). Virus bound (lanes 1, 3) or soluble (lanes 2, 4) gp120 was detected by immunoprecipitation with HIV-1 antibody-positive human serum, SDS-PAGE and autoradiography. In Figure 1B, labeled cells were treated with 0 (lanes 1, 2), 5 μg/ml (lanes 3,4) or 10 μg/ml SC (lanes 5, 6). Virus bound (lanes 1, 3, 5) or soluble (lanes 2, 4, 6) gp120 was detected as in 1A.

Figures 2A and 2B illustrate the susceptibility of gp120 to thrombin digestion in the presence of SC and sCD4. Molt3/HIV-1_{IIIB} cells were labeled with ^{35}S-methionine for 4 hr, followed by a 3 hr incubation with medium containing 0.25% methionine. In Figure 2A, an aliquot of labeled medium (1ml) was digested with thrombin (7 μg/ml) at 37°C for 90 min and then immunoprecipitated with HIV-1 positive human serum and analyzed by SDS-PAGE. Lane 1 shows untreated medium and lane 2, medium treated with thrombin. Prior to thrombin digestion, aliquots of the medium were pretreated with SC at concentrations of 2.5 μg/ml (lane 3), or 10 μg/ml (lane 4); or with sCD4 at concentrations of 2.5 μg/ml (lane 5) or 10 μg/ml (lane 6). The gp120

fragments generated by thrombin cleavage are marked with arrows. In Figure 2B, aliquots of labeled medium were digested by thrombin as before with no pretreatment (lane 1), after pretreatment with 5 μg/ml SC (lane 2 or with a mixture of 5 μg/ml SC and 0.1 mM α-methylpyranoside (lane 3).

Figures 3A and 3B show the inhibition of HIV-1 induced syncytia formation by murine antisera raised against gp120-sCD4. In Figure 3A, murine antiserum raised against gp120-sCD4 was added to CEM cells along with cells infected with HIV-1_{IIIB} (O), HIV-1_{MN} (□) or HIV-2_{WAVZ} (◊). In Figure 3B, murine antisera raised against thrombin treated gp120-sCD4 complexes were tested. The assay conditions are described in the Examples. For each experimental condition, the syncytia in three separate fields were counted. The average value is given as syncytia/field.

Figure 4 shows Western blot assays of monoclonal antibodies raised against gp120-CD4 complexes with gp120, sCD4 and complex. Lane 1 is MoAb 7E3, lane 2 is MoAb 8F10B, lane 3 is MoAb 8F10C, lane 4 is MoAb 8F10D, lane 5 is anti-gp120 MoAb, lane 6 is anti-p24 MoAb (negative control), lane 7 is rabbit anti-CD4 hyperimmune serum, and lane 8 is normal rabbit serum.

Figure 5 is a graph showing the binding of monoclonal antibodies to gp120-lectin complex. MoAbs A (O) and B (Δ) were tested in ELISA, with either gp120-SC (open symbols) or gp120 (closed symbols).

Figure 6 is a graph showing competitive ELISA with monoclonal antibodies and immune goat serum. Limiting dilutions of purified MoAb 7E3 (■), MoAb 8F10B (O), MoAb 8F10C (●) and MoAb 8F10D (▲) were incubated with serial dilutions of goat 69 serum and tested in gp120-CD4 ELISA. Percent competition was calculated as level of antibody binding in immune serum versus binding in prebleed serum.

Figure 7 is a photograph of a gel showing gp120-CD4 complexes prepared according to Example III. In Figure 7, lane 1 is gp120, lane 2 is sCD4, Lane 3 is a gp120-CD4 complex and lane 4 has molecular weight markers.

Figure 8 is a schematic representation of CD4, sCD4 and a fragment of human CD4 containing only the first two domains of human CD4 (referred to as "DID2" in Figure 8). DID2 was prepared as provided in Example VI.

Figure 9 is a schematic representation showing the expression vector PTK13+Neo4 encoding DID2. The sequence of the expression vector PTK13+Neo4 is shown in the sequence listing as SEQ ID NO:3.

Figure 10 is a flowchart showing the purification of DID2 which was prepared as provided in Example V1.

Figure 11A shows a SDS-PAGE profile of DID2 and sCD4 and Figure 11B shows a western blot assay of antibodies raised against DID2 and sCD4. In Figure 11A, lane 1 is DID2, lane 2 is sCD4 and lane 3 has molecular weight markers. In Figure 11B, lane 1 is sCD4, lane 2 is DID2 and lane 3 has molecular weight markers.

Figure 12 shows a SDS-PAGE profile of DID2 and a gp120/DID2 complex prepared according to Example VI. Lane 1 is the gp120/DID2 complex, lane 2 is DID2 and lane 3 has molecular weight markers.

Figure 13 is a graph showing the neutralization of SHIV162P3 virus by serum from rabbits immunized with the gp120/DID2 cross-linked complex.

DETAILED DESCRIPTION OF THE EMBODIMENTS

We determined that it was necessary to unmask or create new epitopes on gp120 and/or CD4 capable of eliciting a strong, broadly neutralizing immune response. We used a covalently linked gp120-CD4 complex as an immunogen. gp120 molecules were covalently coupled to soluble recombinant CD4 using bivalent cross-linking agents to ensure that the integrity of the complexes was maintained during any manipulations. The components of the complex were expected to differ from the free glycoprotein in at least two ways: (I) some epitopes on gp120 and CD4 would be masked by complex formation and (II) cryptic epitopes would become exposed as a result of conformational changes in gp120 and CD4 of the complex. Because these epitopes could play a significant role in viral entry into target cells, antibodies directed against them should inhibit

some aspects of the entry process. We believed these antibodies may not inhibit gp120-CD4 interaction but may instead prevent post-binding fusion events necessary for infection.

The application of this strategy toward anti-HIV vaccines offered several other advantages. First, epitopes specific to complexed gp120 are not expected to be normal targets for neutralizing antibodies in vivo. HIV-1 binds and enters target cells within 3 min at 37°C (26). Given the transient and short-lived nature of the native gp120-CD4 complex, it is unlikely that it is presented to the immune system in such a way as to elicit complex-specific antibodies. Therefore, the absence of immune selection in vivo should in turn be reflected in a minimal degree of variation in the complex-specific epitopes of different viral strains. Second, antibodies against complex-specific epitopes on CD4 are not expected to elicit anti-self antibodies capable of recognizing uncomplexed CD4 on the surface of normal cells. This is especially important, since anti-CD4 antibodies can mediate cytotoxic effects.

In the development of vaccines against HIV, the ability to induce novel epitopes on gp120 in the absence of CD4 would be of considerable advantage. We have discovered that this is possible. We have bound a mannose-specific lectin, SC, with gp120, which induces a conformational change on the glycoprotein that appears to be similar to that observed with sCD4. The alterations include exposure of the V3 loop to exogenous protease and dissociation of gp120 from the virus membrane. Therefore, covalently linked gp120-SC complexes are also useful as immunogens for exposing novel epitopes and complex specific antibodies in the absence of CD4.

Further, complexes of gp120 covalently bound to other CD4 equivalent molecules are also useful as immunogens. Preferably, a complex includes gp120 covalently cross-linked to a CD4 equivalent molecule. "CD4 equivalent molecules" as used herein include any molecule that mimics CD4 in conformation and/or induces a conformational change on HIV-1 gp120 that is similar to that induced by CD4. It is preferable that the molecule that mimics CD4 in conformation is also structurally similar to CD4.

CD4 equivalent molecules that are contemplated for use in an immunogenic complex of the present invention include scorpion toxin-based CD4 mimetic miniproteins. Scorpion toxin-based CD4 mimetic miniproteins have been found to exhibit high affinity interaction with gp120, enhance binding of complex-specific monoclonal antibodies to gp120 and inhibit infection of CD4+ T cells by different HIV-1 isolates. See C. Vita et al., Proc. Natl. Acad. Sci. USA 96, pp. 13091-13096 (1999) and C.S. Dowd et al., Biochemistry 41, pp. 7038-7046 (2002).

We have also discovered that immunogenic complexes which elicit high levels of anti-gp120 and anti-complex antibodies with reduced levels of anti-CD4 antibodies following immunization would be of considerable advantage in the development of vaccines against HIV. To this end, we have covalently bound a fragment of CD4, which only contains the first two domains of CD4, with gp120. This complex of gp120 coupled to a fragment of CD4 presents cryptic epitopes on gp120 and/or the fragment of CD4 which are not present on the uncomplexed molecules. Further, these epitopes elicit neutralizing antibodies and are therefore useful in vaccines and immunotherapy against HIV infection.

The fragment of CD4 can alternatively include either the first domain of CD4, the second domain of CD4, or a combination of the first or second domain of CD4 and the third or fourth domain of CD4. It is preferable that the fragment of CD4 include either the first domain of CD4, the second domain of CD4, or the first and second domains of CD4.

An equivalent of any fragment of CD4 can also be included in an immunogenic complex of the present invention. An "equivalent" of any fragment of CD4 as used herein includes any molecule that mimics the conformation of any fragment of CD4 and which can bind to gp120. Preferably, the equivalent is structurally similar to any fragment or combination of fragments of CD4.

Preferably, the vaccines of the present invention are composed of the complex of either gp120-CD4, gp120-CD4 fragment, gp120-CD4 equivalent molecule or gp120-SC together with an acceptable suspension known in the vaccine art. It is further preferable that an adjuvant be added. The only adjuvant

acceptable for use in human vaccines is aluminum phosphate (alum adjuvant), and therefore preferably the vaccine of the present invention is formulated with an aluminum phosphate gel. See Dolin et al., *Ann Intern Med*, 1991; 114:119-27, which is incorporated herein by reference. The dose of the immunogenic complex for purposes of vaccination is between about 40 μg to about 200 μg per inoculation. An initial inoculation may be followed by one or more booster inoculations. Preferably, the vaccination protocol will be the same as protocols now used in clinical vaccination studies and disclosed in Dolin et al., *supra*, and Reuben et al., *J Acquired Immune Deficiency Syndrome*, 1992; 5: 719-725, also incorporated herein by reference.

It is also contemplated that antibodies raised against the immunogenic complexes of the present invention can be used for passive immunization or immunotherapy. The dosage and number of inoculations of these antibodies will follow those established in the art for immunization or immunotherapy with immunoglobulins.

The complexes or antibodies thereto can also be used in a method for the detection of HIV infection. For instance, the complex, which is bound to a solid substrate or labelled, is contacted with the test fluid and immune complexes formed between the complex of the present invention and antibodies in the test fluid are detected. Preferably, antibodies raised against the immunogenic complexes of the present invention are used in a method for the detection of HIV infection. These antibodies may be bound to a solid support or labelled in accordance with known methods in the art. The detection method would comprise contacting the test fluid with the antibody and immune complexes formed between the antibody and antigen in the test fluid are detected and from this the presence of HIV infection is determined. The immunochemical reaction which takes place using these detection methods is preferably a sandwich reaction, an agglutination reaction, a competition reaction or an inhibition reaction.

A test kit for performing the methods mentioned in the preceding paragraph must contain either an immunogenic complex according to the present

invention or one or more antibodies raised thereto. In the kit, the immunogenic complex or the antibody(ies) are either bound to a solid substrate or are labeled with conventional labels. Solid substrates and labels, as well as specific immunological testing methods are disclosed in Harlow and Lane, "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory, 1988, incorporated herein by reference.

EXAMPLES

We conducted several studies to show that new epitopes could be exposed on gp120 and CD4. These studies also demonstrated that neutralizing antibodies could be raised against gp120 after treatment that altered the conformation of the glycoprotein. We have also demonstrated that a complex of gp120 and a fragment of CD4 elicits an anti-HIV-1 response.

EXAMPLE I

a. Conformational Changes in gp120 Induced by Complex Formation with CD4

We analyzed the release of gp120 from the virus surface under various conditions. Molt3/HIV-1_{IIIB} cells were labeled with 35S-methionine (150 μCi/ml) for 3 hours. The labeled cells were then washed and resuspended in RPMI medium containing cold methionine. The cells were then cultured for 4 hours in the presence of recombinant sCD4 (DuPont). The cell-free supernatant was collected and then passed through a Sephacryl S 1000 column in order to separate virions from free viral proteins. Each of the fractions was treated with detergent, immunoprecipitated with human sera positive for anti-HIV-1 antibodies, and analyzed by SDS-PAGE and autoradiography. The amount of gp120 present in the virus and free viral protein fractions was quantitated by a densitometric scan of the autoradiograph. In accordance with previous studies (17, 18), we observed that treatment of virus with sCD4 clearly resulted in an increased level of gp120 in the free protein fraction and a coincident decrease in

the virus fraction (Fig. 1A), indicating that the conformation of gp120 was altered to dissociate it from the virion.

To further investigate how sCD4 alters the conformation of gp120, we conducted studies on thrombin-mediated cleavage of gp120. Digestion of gp120 by thrombin generates 70 KD and 50 KD products (Fig. 2A). This cleavage takes place at the V3 loop. A monoclonal antibody directed against an epitope within the loop blocks the cleavage completely. The thrombin-mediated cleavage at the V3 loop of gp120 is enhanced after binding with sCD4. This indicates an increased exposure of the V3 loop on the surface of the protein, which renders it more susceptible to protease cleavage.

b. Conformational Changes in gp120 Induced by Complex Formation with Succinyl Concanavalin A

It was previously demonstrated that the incubation of HIV-1 with mannose-specific lectins, such as concanavalin A or succinyl concanavalin A attenuates viral infectivity (27, 28). Incubation of 35S-methionine-labeled gp120 with SC resulted in the enhanced susceptibility of the V3 loop to thrombin digestion (Fig. 2A). This effect was specific, as preincubation of lectin with a-methyl mannoside blocked the enhanced effect completely (Fig. 2B). In addition to increasing the exposure of the V3 loop, interaction of HIV-1 with SC resulted in a dissociation of gp120 from the viral membrane (Fig. 1B). The degree of such shedding was somewhat less than that observed with sCD4. Nevertheless, these studies clearly indicated that sCD4 and SC alter the conformation of gp120, and in a very similar manner.

c. Immunological Properties of Chemically Coupled gp120-CD4 Complexes

We demonstrated that gp120-sCD4 complexes are immunogenic and capable of eliciting HIV-1-neutralizing antibodies. An immunoaffinity procedure was used to purify gp120 from chronically-infected H9/HIV-1_{IIIB} cells. The purified gp120 was then crosslinked to sCD4 (DuPont) using the noncleavable,

water-soluble crosslinker, bis(sulfosuccinimidyl) suberate (BS). Mice were inoculated with the complexes and the immune sera examined for any effect on HIV-induced syncytium formation. Syncytium formation induced by HIV-1_{IIIB} and HIV-1_{MN} infected cells was markedly inhibited by the immune sera. A representative inhibition curve of one immune serum is shown in Fig. 3A. Syncytium formation induced by cells infected with the highly related HIV-2 was also inhibited in the presence of the serum. These results demonstrate that gp120-sCD4 complexes are capable of eliciting broadly neutralizing antisera.

We also inoculated mice with complexes comprised of thrombin-digested gp120 and sCD4. In this case, the gp120 V3 loop was expected to be modified by protease cleavage. Since V3 has been reported to be the neutralizing epitope on gp120, it has been of interest to determine how such cleavage would affect the ability of the complex to elicit neutralizing antibodies. As shown in Fig. 3B, inoculation of mice with thrombin-digested gp120-CD4 complexes elicited antibody capable of blocking syncytium formation induced by the HIV-1_{IIIB} and HIV-1_{MN} isolates. However, this inhibiting effect was not observed with HIV-2 induced syncytium formation.

Our preliminary experiments clearly demonstrated that the covalently coupled gp120-CD4 complexes can elicit a broadly neutralizing antibody response. We then undertook to determine whether cryptic epitopes on the complex are recognized by the neutralizing antibodies and to characterize the epitopes.

<u>EXAMPLE II</u>

a. Immunological Properties of gp120-CD4 Complex

The glycoprotein gp120 used in the preparation of gp120-CD4 complex was purified H9/HIV-1IIIB cells by immunoaffinity chromatography. The cells were lysed in a buffer containing 20 mM Tris (ph 8.2), 0.15 M NaCl, 1.0% Triton X-100, and 0.1 mM PMSF. The lysate was centrifuged at 100,000 x g for 1 hr. The NaCl concentration in the supernatant was adjusted to 1 M and the lysate

was then reacted with an affinity matrix prepared with human anti-HIV immunoglobulins purified from serum of an HIV-antibody positive subject. The bound antigens were eluted with 50 mM diethylamine, pH 11.5, and the pH of the eluate was immediately adjusted to 8.0 with Tris HCl. The eluate was extensively dialyzed against 10 mM phosphate buffer (pH 6.5) containing 0.5 M NaCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM $MnCl_2$, followed by the addition of Triton X-100 to reach 0.2% by weight solution of the detergent. The dialyzed material was then passed through a lentil-lectin column. The glycoproteins were isolated from the lentil-lectin column by elution with 0.4 M α-methylmannoside and were then dialyzed against 20 mM Tris HCl (pH 8.2) containing 1 M NaCl and 0.2% Triton X-100. The dialyzed material was then applied to an affinity matrix prepared with a mouse monoclonal antibody SVM-25 (U.S. Patent 4,843,011) reactive against gp41 to absorb gp160 and any gp41 present. The flow-through from the affinity column was dialyzed extensively against 10 mM BES (pH 6.5) containing 1 mM EDTA and was loaded on a phosphocellulose column equilibrated with the same buffer. The column was developed with a linear gradient of 0-500 mM NaCl and fractions containing gp120 were pooled, concentrated, and dialyzed against PBS.

The purified glycoprotein was coupled to sCD4 (commercially obtained from DuPont) by using bis (sulfosuccinimidyl) suberate (BS) (Pierce) as a crosslinker. For this gp120 and sCD4 were mixed at 1:2 molar ratio in PBS and incubated at 37° C for 1 hr followed by treatment with 0.5 mM BS at room temperature for 1 hr. The complex was further incubated overnight at 4°C. The excess BS was blocked with 20 mM Tris-HCl (pH 8.0).

b. Development of gp120-CD4 Complex-Specific Monoclonal Antibodies

Balb/C mice were subjected to six biweekly inoculations of the gp120-CD4 complex. The initial inoculum (48 μg per mouse) was emulsified in Complete Freunds Adjuvant and administered by subcutaneous injection. In subsequent inocula (24 μg/mouse) were emulsified in Incomplete Freunds Adjuvant and were

administered by intraperitoneal injection. Two weeks after the final inoculation the animals were bled and the sera examined for HIV-1 neutralizing antibodies by a syncytium-blocking assay. Briefly, CEM cells (1 10^5) were cocultured with HIV-1 infected cells ($1x10^4$) in the presence of the test serum and the number of giant cells were counted after 24-40 hr. Syncytium formation induced by HIV-1_{IIIB}- and HIV-1_{MN}-infected cells was markedly inhibited by the serum of the mice that was immunized with gp120-CD4 complex. Syncytium formation induced by HIV-2-infected cells was also inhibited by these sera indicating that gp120-CD4 complexes are capable of eliciting broadly neutralizing antibodies in mice.

After detection of neutralizing antibodies in mice, the animals received a final intraperitoneal form of gp120-CD4 complex in PBS without adjuvant. On the fourth day, the animals were sacrificed and the spleen extracted. Splenic lymphocytes were flushed from the spleen with a syringe. The cells ($7x10^7$) were fused with $1x10^7$ NS-1 mouse myeloma cells (ATCC, Rockville, Maryland), overnight in super HT [DMEM containing 20% fetal calf serum (Hyclone), 0.1 M glutamine, 10% NCTC-^{109} lymphocyte conditioned medium, 0.5 mM Na-pyruvate, 0.2 U/ml insulin, 1 mM oxalacetic acid, and 100 u/ml penicillin/streptomycin] (GIBCO) containing 40% PEG 1540. The cells are then suspended in super HT containing 0.4 μM aminopterin and placed in 96-well plates.

Initially, hybrodomas were selected for the production of gp120-CD4 and gp120-CD4 complex-specific antibodies. Pooled hybridoma supernatants were tested in the ELISA using gp120, CD4 and gp120-CD4 as antigens. Supernatants of pools containing complex-specific antibodies were tested individually. Hybridomas of interest were cloned by replating in super HT at a density of 1 cell/well. Supernatants from cloned hybridomas were further tested by ELISA using gp120-CD4 complexes.

Four hybridomas were selected which secreted immunoglobulin demonstrating a high level reactivity against gp120-CD4 complex and negligible reactivity with either gp120 or sCD4 in ELISA (Table 1). Notably, one of the monoclonal antibodies, MoAb 7E3, was of the IgA isotype. Immunoglobulins were subsequently purified from the ascites fluid of each hybridoma and further

analyzed by Western blot assay with gp120-CD4 complexes, free gp120, or sCD4. While none of the antibodies reacted with free gp120 or sCD4, antibodies 7E3 and 8F10B displayed high levels of reactivity with the complex (Fig. 4) and a low molecular weight fragment of complex. Although antibodies 8F10C and 8F10D reacted strongly with the complex in ELISA (Table 1), reactivity with the complex in Western blot was weak. These results suggest that MoAbs 8F10C and 8F10D are directed against a set of highly conformation-dependent, complex-specific epitopes that are distinct from the epitopes recognized by MoAbs 7E3 and 8F10B.

Purified 7E3, 8F10B, 8F10C, and 8F10D immunoglobulins were tested in cell-free infection assays using PHA-stimulated peripheral blood mononuclear cells (PBMCs) and a variety of HIV-1 isolates. As shown in Table 2, none of the antibodies had any significant effect on the infection of PBMC by the laboratory-adapted strain, HIV-1IIIB. However antibodies 7E3, 8F10B, and 8F10C neutralized the infection of PBMC by a primary isolate of HIV-1MN to a significant extent, whereas antibody 8F10D had no effect. In contrast to these results, none of the antibodies blocked syncytium formation induced by H9/HIV-1IIIB or H9/HIV-1MN on CEM cells. Our preliminary experiments suggest that the extent of cell-free neutralization by these complex-specific antibodies may depend on the infection rate of the isolate. In general, primary HIV-1 strains with lower infection rates tend to be neutralized more effectively than more virulent lab-adapted strains of HIV-1.

To determine whether the complex-specific antibodies bind to the gp120 or the CD4 moiety of the complex, we took advantage of our demonstration that the mannose-specific lectin, succinyl conA (SC), perturbs the conformation of the glycoprotein in a manner similar to that induced by sCD4 (33). SC and gp120 were cross-linked with BS3 and tested in ELISA. MoAbs 7E3 and 8F10B reacted strongly with the gp120-SC complex (Fig. 5) but did not react with free gp120 or SC. In contrast, antibodies 8F10C and 8F10D showed only weak binding to the complex. These results suggest that antibodies 7E3 and 8F10B are directed towards either cryptic epitopes exposed on gp120 in response to SC binding or

new epitopes created in the protein following the chemical reaction with BS3 during crosslinking. Recent immunological characterization of these antibodies has revealed that these antibodies recognize an epitope present in the region of crosslinker BS3 introduced in the gp120 molecule and are not specific to gp120.

c. Immunological Response Against gp120-CD4 Complex in Goats

We have also analyzed the immunogenic response against gp120-CD4 complex in a larger species of animals. An animal (goat 69) was repeatedly inoculated with 100 μg gp120-CD4 complex in Freund's adjuvant and after the fifth inoculation the serum was examined by ELISA for reactivity with gp120, sCD4 and the complex. Antibodies reactive against both free gp120 and sCD4 were detected in the sera. To determine if complex-specific antibodies were also elicited, the serum was tested in cross-competition assays with MoAbs 7E3, 8F10B, 8F10C, and 8F10D. Two-fold serial dilutions of goat 69 serum were incubated with limiting dilutions of each MoAb and tested in gp120-CD4 complex ELISA. As shown in Fig. 6, antibodies in the goat serum were able to block the binding of all four monoclonal antibodies.

The goat serum was tested for neutralizing antibodies in syncytium blocking and cell-free infection assays (Table 3). For comparison, serum from another animal (goat 58) taken after five inoculations with HIV-1IIIE viral gp120, was also tested. In syncytium assays, goat 69 serum reduced syncytium formation ≥ 80% at titers of 1:640 and 1:80 against HIV-1IIIB and HIV-1MN, respectively; goat 58 serum was much less effective. Goat 69 serum neutralized cell-free infection of CEM cells by HIV-1IIIB with a titer of 1:80. Again, this titer was significantly higher than the titer (1:20) of goat 58 serum. Goat 69 serum also mediated group-specific neutralization of cell-free infection by primary isolates HIV-1MN and HIV-1JRFL (Table 3). The neutralizing titer (1:80) was comparable to that of a broadly neutralizing human serum (1:160) tested in parallel; goat 58 serum failed to block HIV-1MN infection even at <1:20 dilution. Goat 69 serum was retested after removal of anti-CD4 antibodies by

preabsorption with CEM cells. Removal of such antibodies was verified by flow cytometric analysis with SupT1 cells which showed nearly 90% reduction in cell surface binding. Despite this reduction, the neutralization titer of the absorbed serum was only two-fold less (1:40) than unabsorbed serum, indicating that neutralization is not entirely due to anti-CD4 antibodies.

The results presented in this example indicate that covalently cross-linked gp120-CD4 complexes possess a number of immunogenic complex-specific epitopes. At least a portion of these epitopes reside on the gp120 moiety of the complex. Moreover, some complex-specific epitopes are targets for broadly neutralizing antibodies specifically effective against cell-free infection by diverse HIV-1 strains, including primary field isolates targeted toward PBMC. Based on these findings, it is possible that the complexes could serve as a protective vaccine or immunotherapeutic reagent.

EXAMPLE III

a. Preparation of gp120-CD4 Complex (1:1 Molar Ratio) Free from Any Uncomplexed CD4

In the immunization protocol described above gp120 and CD4 were complexed at a 1:2 molar ratio. As the immunization with this material resulted in the isolation of anti-CD4 antibodies, we wanted to prepare gp120-CD4 complex (1:1 molar ratio) free from any uncomplexed receptor molecules to optimize the conditions for eliciting anti-gp120 antibodies. gp120 and CD4 (1:1 molar ratio) were bound at 37°C for 1 hr, reacted with BS for 1 hr at room temperature and then overnight at 4°C. After blocking the free crosslinker with Tris buffer (pH 8.0), the solution was treated with Sepharose coupled to anti-CD4 monoclonal antibody E for 30 min at room temperature. As E binds to an epitope on CD4 involved in the interaction with gp120, this treatment removed any uncomplexed CD4 present. A gel showing gp120-CD4 complex prepared in this manner is shown in Figure 4. It was clear that only the complex with molecular weight 170

kD and ~340 kD is evident in the gel. There was no free gp120 or CD4 present in the preparation.

EXAMPLE IV

In order to more accurately determine if the immune response to gp120-CD4 complexes differs from the responses to the individual complex components, the following experiment was conducted. Separate groups of mice were inoculated with equal amounts of CD4, gp120 or gp120-CD4 complexes. After five inoculations, sera were taken from the animals and analyzed. As shown in Table 4, all three of the CD4-immunized animals possessed syncytium blocking seroantibodies effective against HIV-1_{IIIB} and HIV-1_{MN}. All four sera from the complex-immunized animals blocked HIV-1_{IIIB} induced syncytia; two of the four also blocked syncytia induced by HIV-1_{MN}. Overall, neutralizing titers in sera from complex-immunized animals was lower than sera from CD4-immunized animals. Surprisingly, none of the gp120-immunized animals displayed syncytium blocking seroantibodies.

Reactively with CD4 in ELISA between the CD4-immunized and complex-immunized groups was similar (Table 4). The one exception was a complex-immunized animal (mouse 8) which possessed a titer of anti-CD4 antibodies significantly lower than the other animals. Among complex-immunized animals, the level of anti-CD4 reactivity did not correlate with syncytium blocking activity; mouse 10 serum was more effective in blocking syncytia than mouse 9 serum, even though mouse 9 serum had a slightly higher level of anti-CD4 reactivity.

Overall, complex-immunized animals possessed lower titers of anti-V3 loop antibodies; such antibodies were virtually absent from mouse 9 serum.

EXAMPLE V

Sera from CD4-immunized and complex-immunized animals were also tested for reactivity with a variety of synthetic peptides derived from the CD4 sequence (Table 5). Although the overall level of anti-CD4 reactivity between

CD4-immunized and complex-immunized groups was similar (Table 4), the patterns of reactivity with linear epitopes differed. While sera from CD4-immunized animals reacted with peptides derived from the N-terminal portion of CD4 (peptides A and B), such reactivity was absent in sera from complex-immunized animals. This is in accordance with the fact that the N-terminus of CD4 reacts with gp120. The prevalence of reactivity with a peptide derived from domain 3 of CD4 (peptide D) was also reduced among complex-immunized animals relative to CD4-immunized animals. Notably, reactivity with a peptide derived from domain 4 of CD4 (peptide F) was unique to complex-immunized animals 10 and 11.

The data of Examples IV and V, taken together, indicate that the immune response against gp120-CD4 complexes is unique and different from responses to free CD4 and free gp120. Differences in the anti-complex response are reflected in 1) a reduced response against the gp120 V3 loop; 2) a reduced response against linear epitopes in the CD4 N-terminus; 3) an increased response to linear epitopes in CD4 domain 4. It should be noted that the latter epitopes may be hidden in the free CD4 molecule.

According to the present invention, using gp120-sCD4 complexes as immunogens, we have been able to raise HIV-1 neutralizing antibodies that are complex specific. The results we have obtained with these antibodies show that covalently coupled gp120-CD4 complexes possess immunogenic epitopes that are not normally functional in the unbound proteins.

EXAMPLE VI

In this example, we have demonstrated that a complex of gp120 and a fragment of CD4 is immunogenic and elicits HIV-1 neutralizing antibodies. In particular, a fragment of CD4, which only contains the first two domains of CD4 which are involved in gp120 binding, was cloned and expressed in Chinese hamster ovary ("CHO") cells. Figure 8 shows a schematic representation of this fragment of CD4 (shown as "DID2") in comparison to sCD4 (shown as "SCD4")

and CD4. The fragment of CD4 does not contain the third and fourth domains of CD4.

The DID2 fragment was then purified to homogeneity from the supernatant of the CHO cells. The purified DID2 fragment was shown to complex with gp120 and the complex elicited an anti-HIV-1 response in rabbits. Preparation of DID2, immunological reactivity of DID2 and immunogenicity of the gp120-DID2 complex is discussed below in detail.

a. Expression of DID2 in CHO Cells:

A T4-PMV7 plasmid encoding a human soluble CD4 gene was obtained in a bacterial suspension from the NIH AIDS Research and Reference Reagent program. Bacteria from the suspension were streaked on an agar plate containing amphicillin. Colonies were then picked and subjected to large-scale culture for the preparation of a sufficient amount of plasmid DNA. The region of the CD4 gene encoding the first two domains involved in gp120 binding was PCR amplified using the following primers:

5' Primer: 5' sCD4 Hind (Invitrogen) (SEQ ID NO:1)

ATC TGA AAG CTT ATG AAC CGG GGA
(AAG CTT: Hind III Site; ATG: Start)

GTC CCT TTT AG

3' Primer: 3' CD4 (V_1,V_2) Bam (Invitrogen) (SEQ ID NO:2)

ATA AAT GGA TCC TTA GGT GTC GGA GGC
(GGA TCC: BamHI Site; TTA: Stop)

CTT CTG GAA

The PCR amplified CD4 fragment, DID2, was then inserted into an expression vector under a CMV promoter. The resulting expression vector, PTK13+Neo4, is

shown in Figure 9. The sequence of the expression vector PTK13+Neo4 is shown in the sequence listing below as SEQ ID NO:3.

The resulting expression vector, PTK13+Neo4, was transfected into CHO cells and selected initially with G418 and then with G418 and methotrexate. Stable clones were screened for DID2 expression by antigen capture assays and the CHO clone secreting the highest level of DID2 was located. This clone was adapted to grow in serum free medium and subsequently expanded to a 10 liter culture.

b. Purification and Immunological Reactivity of DID2

DID2 was purified from the supernatant of CHO cells by immunoaffinity chromatography using an anti-CD4 monoclonal antibody. Figure 10 is a flowchart showing the steps used in purifying DID2 in detail.

The purified CD4 fragment, DID2, was then analyzed by SDS-PAGE. Figure 11A shows a SDS-PAGE profile of DID2 compared with that of sCD4 (shown as "SCD4" in Figures 11A and 11B). In Figure 11A, lane 1 is purified DID2, lane 2 is sCD4 and lane 3 has molecular weight markers. Both sCD4 and DID2 migrated as a single band corresponding to molecular weights of ~45kD and ~25kD, respectively, which suggests that both proteins were purified to homogeneity with a high degree of purity.

The immunological reactivity of DID2 was then analyzed by a Western blot assay with hyperimmune sera from human sCD4 immunized macaques. Figure 11B shows the Western blot profile of immunological reactivity of both sCD4 and DID2 with anti-CD4 sera. In particular, Figure in 11B, lane 1 is sCD4, lane 2 is DID2 and lane 3 has molecular weight markers. It is clear from this figure that both sCD4 and DID2 reacted strongly with hyperimmune anti-CD4 macaque sera and therefore contain immunologically reactive epitopes.

c. Covalent Cross-linking of DID2 with HIV-1 gp120

The DID2 fragment was then complexed with gp120 from HIV-1 IIIB by covalent cross-linking. In particular, the DID2 fragment was incubated with gp120 for 2 hours at 37°C and then treated with 0.5mM BS3 for 15 minutes at room temperature. The reaction was terminated with 50mM Tris-HCl (pH 8.0) and the complex was purified by chromatography over a column of Sepharose coupled to anti-gp120 antibody (2C6). The complex was then extensively washed and then eluted with 100mM Na_2CO_3. The pH of the eluate containing the complex was then adjusted to 8.0 and the solution was concentrated. This treatment removed any uncomplexed DID2 (free DID2 fragment). The complex and free DID2 fragment were then analyzed by SDS-PAGE. Figure 12 shows a SDS-PAGE profile of the complex and free DID2 fragment. In Figure 12, lane 1 is the complex, lane 2 is the free DID2 fragment and lane 3 has molecular weight markers. It is clear from this figure that DID2 binds efficiently with gp120 and covalent crosslinking of DID2 and gp120 resulted in the formation of both monomeric and multimeric complexes. Purified complex preparation contained undetectable levels of free DID2 fragment.

d. Immunogenicity of the gp120-DID2 Complex in Rabbits

The immunogenicity of the gp120-DID2 complex purified as described above was then examined in rabbits. Two rabbits (C2267 and C2271) were each immunized with 50 μg of the complex in Ribi adjuvant three times at weeks 0, 4 and 8. Sera from each rabbit was collected 2 weeks after each immunization, i.e., at weeks 2, 6 and 10, and analyzed. Antibody titers measured after each immunization by ELISA against the complex are shown in Table 6 below. Antibody titers measured against gp120, sCD4 and DID2 were also taken (and shown also in Table 6) so that it could be determined whether the immune response to the gp120–DID2 complex differed from the responses to the individual complex components. It is clear from Table 6 that the gp120-DID2

complex elicited an antibody response far superior than that of gp120, sCD4 or DID2.

Sera from the complex-immunized rabbits collected at weeks 6 and 10 was then assayed for neutralization of the SHIV162P3 isolate in U373 cells. Sera from both rabbits neutralized the SHIV162P3 virus as shown in Figure 13. As is readily apparent in Figure 13, neutralization was significantly higher with week 10 sera.

Thus, while there have been described what are presently believed to be the preferred embodiments of the present invention, those skilled in the art will realize that other and further embodiments can be made without department from the spirit and scope of the invention, and it is intended to include all such further modifications and changes as come within the true scope of the invention.

TABLE 1
Reactivity of Monoclonal Antibodies Raised
Against gp120-CD4 Complexes in ELISA

Antibody	Isotype	(OD 450nm)		
		CD4	gp120	Complex
7E3	IgA	.427	.340	>3.0
8F10B	IgG_1	.146	.175	1.5
8F10C	IgG_1	.119	.191	>3.0
8F10D	IgG_1	.208	.202	>3.0
anti-gp120	IgG_1	.088	>2.0	>2.0
anti-CD4	IgG_1	>3.0	.103	>3.0

The results shown are with hybridoma supernatants, the same specificities were evident with purified immunoglobulin.

TABLE 2
Neutralization of Cell-Free HIV-1IIIB
and of HIV-1MN Primary Isolate by gp120-CD4
Complex-Specific Monoclonal Antibodies

Antibody Concentration μg/ml	% Inhibition[a]	
	$HIV\text{-}1_{IIIB}$	$HIV\text{-}1_{MN}$
7E3 100	37	88.7
50	55	69.8
25	0	29.8
8F10B 100	26.2	67.2
50	6.2	36.3
25	0	28
8F10C 100	0	75.8
50	0	29
25	0	0
8F10D 100	17	0
Anti-CD4 (control) 50	100	100

aPHA stimulated PBMC (2x105 cells) were infected with either HIV-1IIIB or a primary isolate of HIV-1MN (50 TCID50) for 18 hr in the presence of the indicated amounts of purified antibodies. Cells were then washed and cultured in fresh medium containing the same quantities of antibodies. The p24 content of the supernatant was determined on day 7 and the percent inhibition was calculated relative to control assays carried out in the absence of the antibodies.

TABLE 3

Neutralizing Activity of Sera from Goats Immunized with either gp120-CD4 Complex or gp120

Serum	Immunogen	Syncytium Blocking[a]		Cell-Free Neutralization[b]		
		HIV-1 Strain		HIV-1 Strain/Target Cell		
		IIIB	MN	IIIB/CEM	MN/PBMC	JRFL/PBMC
Goat 69	gp120-CD4 Complex	1:640	1:80	1:80	1:80	1:80
Goat 58	gp120	1:20	<1:20	1:20	<1:20	Not tested
Goat 69 (Cell Absorbed)	gp120-CD4 Complex	<1:25	<1.25	Not tested	1:40	Not tested

[a] HIV-1_{IIIb}-infected H9 cells were incubated with uninfected CEM cells in the presence of two-fold serial dilutions of each serum. The number of syncytia were scored in 3 fields of each well after 24 hr.

[b] Immune and preimmune serum from each goat was diluted 1:10 in culture media. The immune serum was then diluted serially in preimmune serum, thus maintaining a constant serum concentration in all assay wells. Preimmune goat sera and normal human serum did not demonstrate neutralization relative to control assays in which serum was omitted.

The titers shown produced ≥ 80% reaction in syncytia or neutralization relative to matched preimmune sera.

TABLE 4

Mouse	Immunogen	Syncytia Blocking Titer[a] $HIV\text{-}1_{IIIB}/HIV\text{-}1_{MN}$	$HIV\text{-}1_{B10}{}^{V3}$ Peptide ELISA Titer[b]	CD4 ELISA Titer[c]
1	CD4	1:1600/1:1600	Not Tested	>1:256,000
2	CD4	1:800/1:1600	Not Tested	>1:256,000
3	CD4	1:800/1:400	Not Tested	>1:256,000
4	gp120	<1:50/<1:50	1:3200	Not Tested
5	gp120	<1:50/<1:50	1:1600	Not Tested
6	gp120	<1:50/<1:50	1:200	Not Tested
7	gp120	<1:50/<1:50	1:3200	Not Tested
8	complex	1:100/<1:50	1:400	1:32,000
9	complex	1:100/<1:50	<1:25	1:256,000
10	complex	1:400/1:200	1:800	1:128,000
11	complex	1:400/1:100	1:800	1:128,000

[a] Titers are given as the highest serum dilution producing 100% blocking of syncytia formation. Preimmune sera did not reduce syncytia relative to control experiments in which serum was absent.

[b] Serial two-fold dilutions of each serum was tested. ELISA values (absorbance at 450nm) were converted by subtraction of values obtained with the same dilutions of preimmune serum. Titers are given as the highest serum dilution having a corrected ELISA value of ≤ 0.5.

[c] Titers are given as the highest serum dilution having a converted ELISA value of ≤ 0.5.

TABLE 5

CDR4 Peptide ELISA Values (A_{450nm})[a]

Mouse	Immunogen	A	B	C	D	E	F	G	H	I	J	K
1	CD4	1.58	1.05	0.16	2.65	0.21	0.18	0.35	0.21	0.13	0.15	0.19
2	CD4	2.37	0.38	0.17	2.6	0.22	0.17	0.14	0.21	0.15	0.18	0.24
3	CD4	0.56	0.29	0.12	2.34	0.18	0.13	0.21	0.19	0.13	0.14	0.20
8	Complex	0.28	0.27	0.17	0.40	0.23	0.16	0.13	0.19	0.15	0.16	0.26
9	Complex	0.23	0.29	0.20	0.23	0.21	0.19	0.15	0.20	0.14	0.24	0.17
10	Complex	0.17	0.33	0.26	0.61	0.3	1.53	0.17	0.36	0.17	0.35	0.13
11	Complex	0.33	0.43	0.3	2.2	0.36	0.56	0.34	0.36	0.28	0.3	0.20

[a] Sera were tested at a dilution of 1:1000 for reactivity with peptides derived from the CD4 sequence. Peptide A, residues 25-58; B, residues 37-53; C, residues 318-335; D, residues 230-249; E, residues 297-314; F, residues 330-344; G, residues 350-369; H, residues 310-324; I, residues 81-92 (Benzylated); J, residues 81-92; K, irrelevant peptide. ELISA values ≥ two-fold higher than values with irrelevant peptide are shown in Bold type. Reactivity of preimmune serum with the CD4 peptides was the same as with the irrelevant peptide.

TABLE 6

Rabbit	ELISA Titers Measured Against											
	III_Bgp120			sCD4			D1D2			gp120-D1D2		
	Week 2	Week 6	Week 10	Week 2	Week 6	Week 10	Week 2	Week 6	Week 10	Week 2	Week 6	Week 10
C2267	50	1600	102400	25	6400	102400	50	25600	102400	1600	1638400	409600
C2271	200	6400	102400	50	1600	102400	25	6400	102400	800	409600	1638400